

### AMENDMENTS TO THE SPECIFICATION

Please replace the paragraph beginning on page 6, line 8 with the following text:

The amino acids to be produced are not particularly limited so far as the genes concerning the biosynthesis and promoters thereof have been elucidated. Examples of effective enzymes concerning the biosynthesis include GDH, citrate synthase (CS), isocitrate ~~synthase~~ dehydrogenase (ICDH), pyruvate dehydrogenase (PDH) and aconitase (ACO) for glutamic acid fermentation.

Please replace the paragraph beginning on page 6, line 8 with the following text:

The mutation may be caused in either only one promoter sequence such as that for GDH or two or more promoter sequences such as those for GDH, citrate synthase (citrate-synthesizing enzyme) (CS) and isocitrate ~~synthase~~ dehydrogenase (isocitrate-synthesizing enzyme) (ICDH).

Please replace the paragraph beginning on page 9, line 26 with the following text:

Similarly, the mutation can be introduced in the promoter for citrate-synthesizing enzyme (CS) or ~~isocitrate-synthesizing enzyme~~ isocitrate dehydrogenase (ICDH).

The DNA sequence of *icd* gene of coryneform bacterium, which codes ~~citrate synthase~~ isocitrate dehydrogenase, has already been elucidated [J. Bacteriol. 177, 774-782 (1995)]. On the bases of this sequence, primers shown in Seq ID No. 14 and Seq ID No.15 were synthesized. PCR was conducted by using chromosomal DNA of *Brevibacterium lactofermentum* ATCC13869 as the template to amplify about 3 Kbp of DNA fragment containing *icd* gene and promoter thereof. The amplified fragment thus obtained was completely digested with EcoRI, and mixed with that obtained by complete digestion of pHSG399 (Takara Shuzo Co., Ltd.) with EcoRI to conduct the ligation. After the completion of the ligation, the transformation was conducted using competent cells of *E. coli* JM109. The cells were spread on the L-medium plates containing 10 µg/ml of IPTG, 40µg/ml of X-

Gal and 40µg/ml of chloramphenicol. After overnight incubation, white colonies were taken and the transformants was obtained after single colony isolation.

Please replace the paragraph beginning on page 38, line 18 with the following text:

Primers shown in Seq ID No. ~~[[33]]~~ 35 and Seq ID ~~No.34~~ No. 36 were synthesized on the basis of the previously cloned DNA sequences. PCR was conducted by using chromosome of *Brevibacterium lactofermentum* ATCC13869, prepared with a Bacterial Genomic DNA Purification kit (Advanced Genetic Technologies Corp.), as the template under standard reaction conditions described on page 8 of PCR Technology (edited by H. Erlich and published by Stockton Press, 1989) to amplify *pdhA* gene. Among the primers thus synthesized, Seq ID No. ~~[[33]]~~ 35 corresponded to a sequence of base No. 1397 to No.1416 in *pdhA* gene described in Seq ID No. ~~[[32]]~~ 33 in the Sequence Listing. Seq ID No. ~~[[34]]~~ 36 was the complementary strand of the DNA sequence corresponding to the sequence of base No. 5355 to No.5374 in Seq ID No. ~~[[32]]~~ 33 in the Sequence Listing, which was represented from the 5' side.

Please replace the paragraph beginning on page 39, line 11 with the following text:

From the transformed strains, plasmids were prepared by alkali method (*Seibutsu Kogaku Jikken-sho* edited by Nippon *Seibutsu Kogaku kai* and published by *Baifukan*, p. 105, 1992). Restriction enzyme maps of DNA fragments inserted into the vectors were prepared and compared with the restriction enzyme map of *pdhA* gene reported in sequence No. ~~[[32]]~~ 33 of the Sequence Listing. A plasmid containing DNA fragments inserted therein having the same restriction enzyme map as that of *pdhA* gene was named pSFKBPDHA.

Please replace the paragraph beginning on page 41, line 8 with the following text:

The promoter region of *pdhA* gene was presumed from the DNA sequence which had been already elucidated by cloning. As a result, it was supposed to be possible that base No. 2252 to No.2257 and No. 2279 to No. 2284 in Seq ID No. ~~[[32]]~~ 33 in the Sequence Listing

were -35 region and -10 region, respectively. Therefore, primers shown as Seq ID No. ~~[[35]] 37~~ and Seq ID No. ~~36~~ No. 38 in the Sequence Listing were synthesized, and DNA fragments containing promoter region of *pdhA* gene were amplified by PCR method by using chromosomal DNA of *Brevibacterium lactofermentum* ATCC13869 as a template. Among the synthesized primers, Seq ID No. ~~[[35]] 37~~ corresponded to the sequence ranging from base No. 2194 to base No. 2221 in Seq ID No. ~~[[32;]] 33;~~ but the base No. 2198 had been replaced with C, and the base No. 2200 and No.2202 had been replaced with G, and recognition sequence for restriction enzyme *Sma*I had been inserted. Seq ID No. ~~[[36]] 38~~ corresponded to the sequence ranging from base No. 2372 to base No. 2398 in Seq ID No. ~~[[32;]] 33;~~ but base No.2393 and No.2394 had been replaced with G, and the complementary strand of the DNA sequence having a recognition sequence of restriction enzyme *Sma*I inserted therein was represented from the 5'-end. PCR was conducted by using chromosome of *Brevibacterium lactofermentum* ATCC13869, prepared with Bacterial Genomic DNA Purification kit (Advanced Genetic Technologies Corp.), as the template under standard reaction conditions described on page 8 of PCR Technology (edited by H. Erlich and published by Stockton Press, 1989) to amplify the promoter region of *pdhA* gene. PCR product thus obtained was purified by an ordinary method and reacted with restriction enzyme *Sma* I. The fragments were ligated with pNEOL lacking in promoter region of *lacZ* gene which could be replicate in a coryneform bacterium and which had been digested with restriction enzymes *Sma* I, (Example 4 (3) with a Ligation Kit (Takara Shuzo Co., Ltd.). After the transformation with competent cells (Takara Shuzo Co., Ltd.) of *E. coli* JM109, the cells were spread on the L-medium plates (comprising 10 g/l of bactotryptone, 5 g/l of bacto yeast extract, 5 g/l of NaCl and 15 g/l of agar and having pH 7.2) containing 40µg/ml of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and 25µg/ml of kanamycin. After overnight incubation, blue colonies were taken and the transformed strains were obtained

after single colony isolation. From the transformants, plasmids were prepared by alkali method (*Seibutsu Kogaku Jikken-sho* edited by *Nippon Seibutsu Kogaku-kai* and published by *Baifukan*, p. 105, 1992). After sequencing DNA fragments inserted into the vector by an ordinary method, the plasmid containing the DNA fragment inserted therein was named pNEOLBPDHAp<sub>rol</sub>.

Please replace the paragraph beginning on page 42, line 14 with the following text:

Further, primers indicated as Seq ID No. [[37,]] 39, Seq ID No. [[38]] 40 and Seq ID NO. [[39]] 41 in the Sequence Listing were synthesized for constructing plasmids wherein a region supposed to be the promoter site was changed to the consensus sequence of promoters of coryneform bacteria. By using each of the primers and a primer shown in Seq ID No. [[36,]] 38, DNA fragments wherein the promoter region of *pdhA* gene was changed to the consensus sequence were amplified by PCR method by using chromosomal DNA of *Brevibacterium lactofermentum* ATCC13869 as a template. Among the synthesized primers, Seq ID No. [[37]] 39 corresponded to the sequence ranging from base No. 2244 to base No. 2273 in Seq ID No. [[32;]] 33; base No. 2255 had been replaced with C, and base No. 2257 had been replaced with A; thus only -35 region had been changed to the consensus sequence of the coryneform bacteria. Seq ID No. [[38]] 40 corresponded to the sequence ranging from base No. 2249 to base No. 2288 in sequence No. [[32;]] 33; base No. 2279 and No. 2281 had been replaced with T; thus only -10 region had been changed to the consensus sequence of the coryneform bacteria. Sequence No. [[39]] 41 corresponded to the sequence ranging from base No. 2249 to base No. 2288 in Seq ID No. [[32;]] 33; base No. 2255 had been replaced with C, base No. 2257 had been replaced with A, and base No. 2279 and No. 2281 had been replaced with T; thus both -35 region and -10 region had been changed to the consensus sequence of the coryneform bacteria. PCR was conducted by using chromosome of *Brevibacterium lactofermentum* ATCC13869, prepared with a Bacterial Genomic DNA

Purification Kit (Advanced Genetic Technologies Corp.), as the template under standard reaction conditions described on page 8 of PCR Technology (edited by H. Erlich and published by Stockton Press, 1989) to amplify the promoter region of pdhA gene with these primers so that the promoter region was changed to the consensus sequence. PCR products thus obtained were purified by an ordinary method and reacted with restriction enzyme SmaI. The fragments were ligated with pNEOL lacking the promoter region of lacZ gene, which could replicate in a coryneform bacterium and which had been cleaved with restriction enzymes Sma I, with a Ligation Kit (Takara Shuzo Co., Ltd.). After the transformation with competent cells (Takara Shuzo Co., Ltd.) of E. coli JM109, the cells were spread on the L-medium plates (comprising 10 g/l of bactotryptone, 5 g/l of bacto yeast extract, 5 g/l of NaCl and 15 g/l of agar and having pH 7.2) containing 40 µg/ml of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and 25 µg/ml of kanamycin. After overnight incubation, blue colonies were taken and the transformed strains were obtained after single colony isolation. From the transformed strains, plasmids were prepared by the alkali method (*Seibutsu Kogaku Jikken-sho* edited by *Nippon Seibutsu Kogaku kai* and published by *Baifukan*, p. 105, 1992). After sequencing DNA fragments inserted into the vector by an ordinary method, the plasmid containing DNA fragments, wherein only the sequence in -35 region had been changed to the consensus sequence, inserted therein was named pNEOLBPDHAp35; the plasmid containing DNA fragments, wherein only the sequence in -10 region had been changed to the consensus sequence, was inserted therein was named pNEOLBPDHAp10; and the plasmid containing DNA fragments, wherein the sequences in both -35 region and -10 region had been changed to the consensus sequence, was inserted therein was named pNEOLBPDHAp3510.

Please replace the paragraph beginning on page 45, line 1 with the following text:

Primers shown in Seq ID No. ~~[[40]]~~ 42 and Seq ID ~~No.41~~ No. 43 were newly synthesized on the basis of the DNA sequence which had already been cloned. Among synthesized primers, Seq ID No. ~~[[40]]~~ 42 was the complementary strand of the DNA sequence corresponding to a sequence ranging from base No. 2491 to base No. 2521 in Seq ID No. ~~[[32,]]~~ 33, which was represented from the 5'-end, and to which a sequence comprising three A's followed by four T's at the 5' terminal. Seq ID No. ~~[[33]]~~ 35 was the complementary strand of the DNA sequence corresponding to the sequence ranging from base No. 5020 to base No. 5039 of pdhA gene in Seq ID No. ~~[[32,]]~~ 33, which was represented from the 5'-end. PCR was conducted by using Seq ID No. ~~[[33]]~~ 35 and Seq ID ~~No.40~~ No. 42 as the primers and chromosome of *Brevibacterium lactofermentum* ATCC13869, prepared with Bacterial Genomic DNA Purification Kit (Advanced Genetic Technologies Corp.), as a template under standard reaction conditions described on page 8 of PCR Technology (edited by H. Erlich and published by Stockton Press, 1989). Further, PCR was conducted by using Seq ID No. ~~[[39]]~~ 41 and Seq ID No. ~~[[41]]~~ 43 and chromosome of *Brevibacterium lactofermentum* ATCC13869 as a template. The PCR products thus obtained were purified by an ordinary method. PCR was conducted by using PCR products obtained by using Seq ID No. ~~[[33]]~~ 35 and ~~[[No.40,]]~~ No. 42, PCR products obtained by using Seq ID No. ~~[[39]]~~ 41 and Seq ID ~~No.41~~ No. 43 and Seq ID No. ~~[[33]]~~ 35 and ~~[[41]]~~ 43 as the primers. The PCR condition was as follows: The concentration of these four DNA would be 10  $\mu$ M in the reaction cocktail and La taq (Takara Shuzo Co., Ltd.) was used without template. PCR products were purified by an ordinary method, and reacted with restriction enzyme Sal I and Xho I. The fragments thus obtained were ligated with fragments obtained by digesting temperature-sensitive plasmid pSFKT2 with SalI, which can replicate in a coryneform bacterium, by using Ligation Kit (Takara Shuzo Co., Ltd.). After the transformation with competent cells (Takara Shuzo Co., Ltd.) of *E. coli* JM109, the cells was

spread on the L-medium plates (comprising 10 g/l of bactotryptone, 5 g/l of bacto yeast extract, 5 g/l of NaCl and 15 g/l of agar and having pH 7.2) containing 10 µg/ml of IPTG (isopropyl-β-D-thiogalactopyranoside), 40 µg/ml of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and 25 µg/ml of kanamycin. After overnight incubation, white colonies were taken and transformants were obtained after single colony isolation. From the transformants, plasmids were prepared by the alkali method (*Seibutsu Kogaku Jikken-sho* edited by *Nippon Seibutsu Kogaku-kai* and published by *Baifukan*, p. 105, 1992). After sequencing DNA fragments inserted into the vector, the base sequence was compared with that of *pdhA* gene reported in sequence No. [[32.]] 33. The plasmid containing DNA fragments, wherein only the sequences in -35 region and -10 region of the promoter were changed to the consensus sequence of the coryneform bacteria, inserted therein was named pSFKTPDHApr3510.

Please replace the paragraph beginning on page 46, line 10 with the following text:

A plasmid wherein -35 region of the promoter of *pdhA* gene had been changed to the consensus sequence of coryneform bacteria, and also plasmid wherein -10 region of the promoter of *pdhA* gene had been changed to the consensus sequence of coryneform bacteria were constructed in the same manner as that described above except that Seq ID No. [[39]] 41 in the Sequence Listing was replaced with Seq ID No. [[37]] 39 and [[38.]] 40, respectively. These plasmids were named pSFKTPDHApr35 and pSFKTPDHApr10, respectively.

Please replace the paragraph beginning on page 48, line 23 with the following text:

In order to amplify *argG* gene of *Brevibacterium flavum* by PCR, the DNA sequences in the upstream and downstream regions of the ORF were determined. The determination of the DNA sequences was conducted by synthesizing a primer based on the known DNA sequence (Gen Bank accession AF030520) of ORF of *argG* gene of *Corynebacterium glutamicum* and using in vitro LA PCR cloning kit (Takara shuzo Co., Ltd.) in accordance

with the instruction manual included in the kit. As primers, they were specifically used ~~oligonucleotide~~ oligonucleotides (primers 1 and 2) having the DNA sequences set out as Seq ID No. ~~[[42]] 44~~ and Seq ID ~~No.43~~ No. 45 for the upstream region, and ~~oligonucleotide~~ oligonucleotides (primers 3 and 4) having the DNA sequences set out as Seq ID ~~No.44~~ No. 46 and Seq ID ~~No.45~~ No. 47 for the downstream region. The DNA sequences in the upstream and downstream region of argG were determined by completely digesting chromosome DNA of 2247 strain (ATCC14067), i.e., wild type strain of *Brevibacterium flavum*, with a restriction enzyme EcoRI, conducting first PCR with the primer 2 or 3 (having sequence No. ~~[[43]] 45~~ or ~~[[44]],~~ 46), and conducting second PCR with the primer 1 or 2 (having sequence No. ~~[[42]] 44~~ or ~~[[45]].~~ 47).

Please replace the paragraph beginning on page 49, line 18 with the following text:

Mutation-introducing primers 9, 10, 11, 12 or 13 and 7 (having sequence ~~No. 50, 51, 52, 53, 54 or 48, respectively~~ No. 52, 53, 54, 55, 56, or 50, respectively) for a region of the highest score were used, and the first PCR was conducted with chromosomal DNA of AJ12092 strain as a template. The second PCR was conducted with the same chromosomal DNA as the template by using the PCR product as the primer for 3'-end and also using the primer 8 having sequence No. ~~[[49]] 51~~ as the primer on 5'-end to obtain DNA fragments having the mutation introduced in the intended promoter region. To determine the activity of the mutant promoters, these DNA fragments were inserted into SmaI site of promoter probe vector pNEOL so that they were in the same direction with lacZ reporter gene to obtain plasmids pNEOL-1, pNEOL-2, pNEOL-3, pNEOL-4 and pNEOL-7. As a control for the activity, plasmid pNEOL-0 was constructed by inserting the DNA fragment, obtained by PCR using chromosomal DNA of AJ12092 strain and primers 7 and 8, into the upstream of lacZ gene of pNEOL.

Please replace the paragraph beginning on page 51, line 6 with the following text:



PCR was conducted by using primers 14 and 15 (having the sequence of Seq ID No. ~~[[55]] 57~~ and Seq ID ~~No. 56~~ No. 57) with chromosomal DNA of AJ12092 strain as the template. These DNA fragments thus obtained were inserted into a *Sma*I site in a multicloning site of cloning vector pHSG398 (a product of TaKaRa) to construct plasmid p0. Then, p0 was digested with restriction enzymes EcoRV and BspHI, and also pNEOL-3 and pNEOL-7 were digested with restriction enzymes EcoRV and BspHI. DNA fragments thus obtained were ligated to obtain mutation-introducing plasmids p3 (mutant derived from mutation-introducing primer 11) and p7 (mutant derived from mutation-introducing primer 13).

Please replace the paragraph beginning on page 52, line 2 with the following text:

Based on the DNA sequence determined as in (1), oligonucleotides (primers 5 and 6) having the DNA sequence set out in Seq ID No. ~~[[46]] 48~~ and Seq ID ~~No. 47~~ No. 49 were synthesized to conduct PCR using chromosomal DNA of *Brevibacterium flavum* as a template. The PCR reaction was conducted in 25 cycles, each cycle consisting of 94°C for 30 seconds, 55°C for one second and 72°C for 2 minutes and 30 seconds. The thus-obtained DNA fragment was cloned to *Sma*I site in multi-cloning site of cloning vector pSTV29 (Takara shuzo Co. Ltd.) to obtain pSTVargG. Furthermore, pargG was prepared by inserting into *Sal*II site of pSTVargG a fragment containing the replication origin obtained by treating pSAK4 set out in Example 1 with *Sal*II.

Please replace the paragraph beginning on page 54, line 21 with the following text:

Plasmids having GDH promoter sequence of FGR1 strain and FGR2 strain described in Example 2 were constructed by site directed mutagenesis. For obtaining GDH promoter sequence of FGR1 strain, PCR was conducted by using synthetic DNA shown in Seq ID No. ~~[[57]] 59~~ and synthetic DNA shown in No. ~~[[60]] 62~~ as the primers and chromosomal DNA of ATCC13869 as the template; and on the other hand, PCR was conducted by using

synthetic DNA shown in Seq ID No. [[58]] 60 and synthetic DNA shown in Seq ID No. [[59]] 61 as the primers with chromosomal DNA of ATCC13869 as the template. Further, PCR was conducted by using synthetic DNAs shown in Seq ID Nos. [[57]] 59 and Seq ID ~~No. 58~~ No. 60 as the primers with a mixture of these PCR products as the template. The PCR product thus obtained was inserted into SmaI site of pSFKT2 (Japanese Patent Application No. 11-69896) to construct pSFKTG11. To obtain GDH promoter sequence of FGR2 strain, PCR was conducted by using synthetic DNA shown in Seq ID No. [[57]] 59 and synthetic DNA shown in Seq ID No. [[62]] 64 as the primers and chromosomal DNA of ATCC13869 as the template; and on the other hand, PCR was conducted by using synthetic DNA shown in Seq ID No. [[58]] 60 and synthetic DNA shown in Seq ID No. [[61]] 63 as the primers and chromosomal DNA of ATCC13869 as the template. Further, PCR was conducted by using synthetic DNA shown in Seq ID No. [[57]] 59 and Seq ID No. [[58]] 60 as the primers and a mixture of these PCR products as the template. The PCR product thus obtained was inserted into SmaI site of pSFKT2 (Japanese Patent Application No. 11-69896) to construct pSFKTG07. The DNA sequences of the fragments inserted into SmaI sites of pSFKTG11 and pSFKTG07 were determined to confirm that no mutation was introduced into other regions than the promoter region in GDH.

Please replace the paragraph beginning on page 56, line 19 with the following text:

PCR reaction was conducted by using synthetic DNA shown in Seq ID No. [[63]] 65 and Seq ID ~~No. 64~~ No. 66 as the primers and chromosomal DNA of wild-type coryneform bacterium strain ATCC13869 as the template. The *gdh* gene fragment thus obtained was inserted in Ball site of pAJ220 to construct pAJ220G. The promoter was present near Ball site of pAJ220, and the expression of the inserted gene was increased depending on the direction of the gene inserted into Ball site. PAJ220G and pGDH were introduced into ATCC13869 strain by electrical pulse method. GDH activities of the strains thus constructed

were determined by the method stated in above-described step (1). As a result, GDH activity of the strain into which pAJ220G had been introduced was about 1.5 times as high as that of the strain into which dGDH had been introduced as shown in Table 24.

Please delete the original Sequence Listing appearing on pages 60-88.

Page 93 (Abstract), after the last line, beginning on a new page, please insert the attached substitute Sequence Listing.